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Thomas W. O'Neil

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Butler University Botanical Studies

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Edited by

J. E. Potzger

The *Butler University Botanical Studies* journal was published by the Botany Department of Butler University, Indianapolis, Indiana, from 1929 to 1964. The scientific journal featured original papers primarily on plant ecology, taxonomy, and microbiology. The papers contain valuable historical studies, especially floristic surveys that document Indiana's vegetation in past decades. Authors were Butler faculty, current and former master's degree students and undergraduates, and other Indiana botanists. The journal was started by Stanley Cain, noted conservation biologist, and edited through most of its years of production by Ray C. Friesner, Butler's first botanist and founder of the department in 1919. The journal was distributed to learned societies and libraries through exchange.

During the years of the journal's publication, the Butler University Botany Department had an active program of research and student training. 201 bachelor's degrees and 75 master's degrees in Botany were conferred during this period. Thirty-five of these graduates went on to earn doctorates at other institutions.

The Botany Department attracted many notable faculty members and students. Distinguished faculty, in addition to Cain and Friesner , included John E. Potzger, a forest ecologist and palynologist, Willard Nelson Clute, co-founder of the American Fern Society, Marion T. Hall, former director of the Morton Arboretum, C. Mervin Palmer, Rex Webster, and John Pelton. Some of the former undergraduate and master's students who made active contributions to the fields of botany and ecology include Dwight. W. Billings, Fay Kenoyer Daily, William A. Daily, Rexford Daudenmire, Francis Hueber, Frank McCormick, Scott McCoy, Robert Petty, Potzger, Helene Starcs, and Theodore Sperry. Cain, Daubenmire, Potzger, and Billings served as Presidents of the Ecological Society of America.

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A SIMPLIFIED MEDIUM FOR USE IN TISSUE CULTIVATION OF POLIOMYELITIS VIRUS

THOMAS W. O'NEIL
Eli Lilly and Company
Indianapolis, Indiana

Dr. John Enders' (1) outstanding discovery in 1949 that all three types of Poliomyelitis virus would grow in tissue culture of extraneural cells, has paved the way for Dr. Jonas Salk's success in developing a safe and effective Poliomyelitis virus vaccine. Although virus vaccines are not new, this vaccine developed by Dr. Salk is the first to be produced on a large scale in tissue culture for human use. The advances made against this dreaded disease were made possible by the improvements in tissue culture techniques. One of the more important advances has been the development of a synthetic medium known as Medium 199 (2). Although this medium has proved satisfactory in supporting the growth of tissue for the cultivation of Poliomyelitis virus, the expense and the limited supply of the large number of amino acids, nucleic acids, and vitamins of which the medium is composed, and the complexity of its preparation has made this medium costly and tedious to produce.

This study was undertaken with the hope of finding a simpler medium for the tissue cultivation of Poliomyelitis virus for use in vaccines.

REVIEW OF LITERATURE

The advance made in Poliomyelitis research has coincided with the development of tissue culture media and improvements in tissue culture techniques. Since the classical observation of the *in vito* growth of embryonic neural tissue by R. J. Harrison (3), volumes of work have accumulated on procedures that permit the survival or growth of cells outside the body. A variety of media have been used for maintenance and growth of tissues. The monograph of Parker (4) gives an extensive description of techniques used in tissue culture as well as media that allow for the growth of cells.

The media employed for the growth of tissue cultures prior to 1950 have been derived directly or indirectly from the organism and consist of blood plasma, blood serum, embryo tissue extract, body exudates, and extracts of varions tissues and organs mixed with a physiological salt solution. Though such media are unsatisfactory for cultivating virus for vaccine purposes and for studying the nutritional requirements of the cell, the studies on the nutrition of the cell are worth mentioning for the information these papers give on substances found to have cell growth stimulating properties.

Lewis and Lewis (5) were among the first to study the behavior of cells

in media of known composition. In 1911, they investigated the survival of chick embryo tissues in solutions containing varying concentrations of sodium chloride, calcium chloride, potassium chloride and sodium bicarbonate, and found that growth was influenced to a marked degree by variations in the proportion of these salts. They also found that excellent growth could be obtained in solutions of amino acids and polypeptids of known composition and that media containing dextrose or maltose were better than pure salt solutions. Unfortunately, no precise details of these media were supplied. Later, work by Burrows and Neyman (6) indicated that hydrolytic products of egg yolk, and mixtures of ten amino acids were toxic for chick embryo cells. From this work the concept that amino acids are toxic for tissue cultures has persisted until recent times.

Carrel (7) in 1913, focused attention upon the role of proteins in cell nutritions by noting that extracts of tissues and particularly embryo tissues greatly accelerated the growth of fibroblasts. Carrel and Baker (8) in 1926, discovered that fibroblasts, epithelial cells, and monocytes, when cultivated in plasma, obtain their nitrogen from proteoses, and possibly other split products of the protein. They also found that peptic digests of egg white and fibrin which contained large amounts of proteoses and peptones, stimulated the cells to greater activity than did tryptic digests which consisted almost entirely of the lower, less complex, hydrolytic products of proteins. They concluded that the cells do not feed on amino acids and other ultrafilterable constituents of embryo extracts, but rely on proteins or closely related fractions. Commercial peptones gave variable results, although Witts' peptones appeared suitable. In 1928, Baker and Carrel (9) fractionated Witts' peptone and showed that alpha and beta proteoses were equal as growth promoting substances and they compared the liver hydrolysates of pepsin, trypsin, and hydrochloric acid. They noted that the products of the peptic digest were more stimulating to growth of the cells than were the other digests prepared with trypsin and hydrochloric acid. They also showed that amino acids contribute to the growth of cells, but are unable to maintain the viability of the cell without the addition of peptids or polypeptids to the medium. In another report of the same year Baker and Carrel (10) further established that the pepsin hydrolytic products of crystalline egg albumin, purified casein, crystalline edestin and purified fibrin are utilized by fibroblasts for their proliferation. It might be noted that the above products were used in combination and no success was observed when any of the hydrolytic products were used alone. The results of Baker and Carrel warrant their conclusion that protein degradation products alone do not provide an adequate diet for all types of cells; the presence of plasma, embryo extract or serum is also necessary. This supplementary action of complex natural substances has been attributed to the presence of enzymes that slowly digest the proteoses to utilizable amino acids.

In 1936, Baker (11) developed a "feeding solution" for fibroblasts and epithelial cells which is noteworthy in that he recognized the importance of a vitamin supplement. Vitamin A, ascorbic acid, Vitamin D, glutathione, and 10 per cent serum were shown to be beneficial for growth of fibroblasts and epithelial cells. The addition of a Vitamin B complex aided in the cultivation of Monocytes. In 1939, Baker and Ebeling (12) attempted to devise a fluid that would maintain cells without promoting growth. The fluid adopted was similar to the "feeding solution" of 1936, with the addition of ten other substances which included two hormones. Cultured strains of fibroblasts were maintained in a viable condition and with little or no proliferation for periods from forty-three to fifty-six days.

Fischer and his associates (13) published a monograph in 1946, summarizing their work of twenty years on cell nutrition. Fractionation studies on embryo tissue juice revealed that the growth promoting activity was associated with a labile substance of high molecular weight. It was found to occur in the nucleoprotein fraction and was similar to the fraction reported by Baker and Carrel. Fischer believed that the mechanism of action of this substance on cells was a catalytic one. Dialysis of plasma, serum, or embryo extract against ringer-glucose solution resulted in the loss of low molecular weight substances and such substances were found essential for the metabolism of the cells. On the supposition that dialyzed media would be deficient in free amino acids, he investigated the ability of different amino acid solutions to compensate for the substances lost by dialysis. In these studies, Fischer observed that cystine, lysine, and glutamic acid were indispensable for the metabolism of the cells.

Astrup, Fischer, and Volhert (14) studied the distribution of substances in animal and vegetable tissues that could be substituted for the dialyzable constituents of serum or embryo juice and found that active growth stimulants could be obtained from kidney, yeasts, barley malts, and calf embryo muscle.

Fischer and his associates (15, 16) using all substances known to be of biological importance developed two mixtures to which were added dialized plasma, embryo extract, and certain inorganic salts. By deletion of individual amino acids, he attempted to determine which were essential for growth of in vitro cells. Fischer arranged the amino acids in the order of their decreasing importance in growth as cystine, arginine, tryptophane, glutamine, and lysine, and also employed crystalline trypsin and pepsin to prepare digests of pure lactoglobulin and bovine serum albumin. He then devised mixtures of pure amino acids in the proportions found in these proteins and compared the cell growth that was obtained. Invariably the protein digests caused a much greater growth response than did the amino acid mixtures. Fischer concluded that tissue cultures may require an unidentified growth promoting factor of peptid or polypeptid nature.

In 1946, a synthetic medium was devised by White (17) consisting entirely of constituents of known composition in Locke's Solution in which chick embryo tissues were grown in roller tubes directly on the surface of the glass. By this method complex materials such as plasma, serum, and embryo extract were eliminated and the effect of various mixtures of chemically known growth substances were tested directly. White reported this medium maintained skeletal fibroblast of eight day chick embryos in good condition for fifty-eight days and kept heart muscle beating for forty-four days. White's Medium did not contain glutamic acid or cystine, which were the two amino acids found by Fischer to be so important in cell nutrition.

In 1950, Morgan, Norton, and Parker (2) reported on a synthetic medium called Medium 199. Initially a basal solution of casein hydrolysate in Earl's Salt Solution was tried, but proved to be unsuccessful and was replaced with an amino acid basal solution. By a gradual building up process these investigators arrived at Medium 199. This medium was composed of 20 different amino acids, 17 vitamins, 2 lipids, 10 nucleic acid derivatives, phenol red indicator, glucose and a number of inorganic salts. Tissues from the leg muscles of eleven day old chick embryos were cultivated directly on the surface of the glass in roller tubes. In contrast to White, these investigators reduced the amount of tissue in the roller tubes to a minimum in order to eliminate as far as possible the carryover of nutrient substances from the embryo. They reported cells proliferated and cultures remained in good condition for an average period of four to five weeks.

Although no wholly adequate synthetic medium for tissue culture has been devised, this medium of Morgan, Norton, and Parker represents the most complete one devised up to this time. Medium 199 has proved satisfactory for the tissue cultivation of Poliomyelitis virus (18, 19, 20).

Morgan, Morton, and Parker (2), employing Medium 199 as a basal solution, studied the effect of the supplementary addition of serum and embryo extract. They observed that the addition of serum from 0.1 to 20 per cent, did not significantly improve the growth promoting qualities of the synthetic mixture, but the addition of embryo extract, at concentrations of 0.25 to 5.0 per cent, proved highly beneficial and resulted in culture growths comparable to those obtained in media comprised of serum and embryo extract. It was concluded that some factor or factors present in embryo extract were still missing from this synthetic medium.

In 1953, Evans, Shannon, Earl, and Sanford (21) attempted to ascertain whether either White's or Medium 199 could be used as a basal nutritional medium to maintain suspended mammalian cells (mouse fibroblastic cells). These two media were compared with the following: (1) EBS (Earl's Balanced Salt Solution), (2) EBS and horse serum, and (3) EBS, horse serum, and chick embryo extract. Their data indicated that White's Medium and

Medium 199 were not adequate basal media since neither could maintain mammalian cells.

In another series of experiments, Sanford, Evans, and Earl (22) demonstrated that the protein fraction of chick embryo extract and the ultrafilterable non-protein fraction of horse serum can be omitted from the serum-extract medium without reducing the cell proliferation if the remaining two fractions are supplemented by certain amino acids. Using Medium 199 and different portions of this medium as supplements, it was demonstrated that the amino acids and possibly the vitamin portions were the essential materials in Medium 199.

In 1952, Melnick and Riordan (23) found that the protein containing substances of the serum-embryo extract culture medium could be replaced by a lactalbumin hydrolysate. When lactalbumin hydrolysate is used instead of embryo extracts in nutrient medium, fibroblasts from monkey testicular tissue plasma cultures grow out rapidly. They also successfully cultivated Poliomyelitis virus in a medium composed of 0.5 per cent lactalbumin hydrolysate, Simms' Serum Ultrafiltrate and Earl's Balanced Salt Solution.

Bazely and Rotundo (24) in 1954, showed that human plasma fractions IV and V stimulated monkey kidney cell multiplication and could replace whole serum as a supplement to Medium 199. Yields of Poliomyelitis virus were increased.

Ginsberg and associates (25) in 1955, studied in HeLa Cell cultures the supplementary effects of various broths used for the cultivation of bacteria in a maintenance solution consisting of horse serum or ascitic fluid and balanced salt solution. Their results showed stimulation of cell multiplication with tryptose phosphate broth. These investigators believed that the stimulating supplementary effect of tryptose phospate broth was due to peptones, proteoses, or peptids.

MATERIALS AND METHODS

Seed. The organism used in this study was the Mahoney strain of Type 1 Poliomyelitis virus No. 2171 received from Eli Lilly and Company. The original source of the seed was Dr. Jonas Salk. It was stored in 1 cc. quantities in 2 cc. rubber stoppered glass ampules and held at $-20°C$. One seed passage was used throughout the study. The virus titer of the seed was $10^{-6.6}TCID_{50}$.

Glassware and Equipment. All glassware and equipment were carefully cleaned with detergents and rinsed several times with distilled water according to current culture practices. Sterilizing filters were chemically cleaned and rinsed with several changes of distilled water. Each filter was checked carefully with the "Bubble Test" of Rivers and Mudd (26) to assure sterilizing efficiency.

Medium Preparation. All media were made up in triple distilled pyrogen free water. All chemicals in these studies were reagent grade. All media and solutions were sterilized by filtration using Selas filters. The medium preparation is divided into four sections:

A. Preparation of Milk Hydrolysates
B. Preparation of Medium 199
C. Preparation of Maximum Test Medium
D. Preparation of Minimum Test Medium

A. *Preparation of Milk Hydrolysates.* The milk hydrolysates were selected for testing because it was noted that their amino acid composition is similar to that of Medium 199. A publication of the National Academy of Science Nutritional Research Council (27) and Sheffield Farms Inc. (28) supplied this information. The commercial products tested were selected on the basis of availability. The milk hydrolysates were made up to 10 per cent concentrations in Hank's Salt Solution.

The table which follows lists the milk products tested:

HYDROLYZED MILK PROTEINS TESTED

Commercial Product	Kind of Product	Per Cent Amino Nitrogen to Total Nitrogen
Hycase	Acid Hydrolysate of Casein (mostly amino acids and peptids)	61 %
Hycase Salt Free	Acid Hydrolysates of Casein with salts and vitamins removed (mostly amino acids and peptids)	60 %
N-Z Amine Type A	Pancreatic Digest of Casein (mostly amino acids and peptids)	53 %
N-Z Amino Type B Batch	Pancreatic Digest of Casein (mostly peptones)	39.4 %
Edamin	Pancreatic Digest of Lactalbumin (mostly amino acids and peptids)	58 %
Peptonized Milk	Trypsin Digest of Fat Free Milk (mostly peptones and polypeptids)	33.9 %
Bacto-Casitone	Pancreatic Digest of Casein (mostly amino acids and peptids)	——
Bacto-Casamino	Acid Hydrolysate of Casein (mostly amino acids and peptids)	——
N-Z Case	Tryptic Digest of Casein (mostly peptones)	40.2 %

B. *Preparation of Medium 199.* As stated previously, this medium is composed of twenty different amino acids, seventeen vitamins, two lipids, ten nucleic acid derivatives, phenol red indicator, glucose, and a number of inorganic salts in fourteen different solutions. Morgan, and Parker's (2) procedure for making this medium is followed. All solutions except Numbers 1, 2, and 9 are stored at 4°C. for a period not over thirty days. Solution No. 1, the amino acid fraction, is made fresh each time the medium is prepared. Solutions 2 and 9 form precipitates at 4°C. and thus were stored at room tmperature.

The fourteen solutions of which Medium 199 is composed are:

Solution No. 1. The Amino Acid Fraction (containing seventeen amino acids) and Balanced Salt Solution
Solution No. 2. Tyrosine and Cystine Solution
Solution No. 3. The Vitamin B Solution
Solution No. 4. The Vitamin C, Glutathione and Cysteine Solution
Solution No. 5. Biotin Solution
Solution No. 6. Folic Acid Solution
Solution No. 7. Vitamin A, D, K, Cholesterol and Tween 80 Solution
Solution No. 8. Vitamin E Solution
Solution No. 9. Adenine Sulfate Solution
Solution No. 10. Purine and Pyrimidine Solution
Solution No. 11. Ribose and Desoxyribose Solution
Solution No. 12. Muscle Adenylic Acid Solution
Solution No. 13. Adenosine Triphosphate (ATP) Solution
Solution No. 14. Ferric Nitrate Solution

C. *Maximum Test Medium.* This medium is similar to Medium 199 with the exception that the amino acid fraction is replaced with various concentrations of Edamin, a hydrolysate of lactalbumin. Concentrations of 0.1, 0.2, 0.3, 1.0, 3.0, 7.0, 10.0, 20.0 and 30.0 g/l of Edamin were tested.

D. *Minimum Test Medium.* This medium, unlike Medium 199, was made to consist of various concentrations of Edamin and Hank's Balanced Salt Solution without sodium bicarbonate. Concentrations of 1.0, 5.0 and 10.0 g/l were tested.

Solution Preparation. Reagent grade chemicals were used in all solutions, the latter being sterilized by filtration through Selas filters.

Hank's Solution. The solution was adjusted to pH 7.0 with sodium hydroxide and not buffered as in the original medium with sodium bicarbonate. Concentrations are the same as recommended by Hank and Wallace (29).

Trypsin Buffer. This solution is the phosphate buffered saline of Dulbecco and Vogt (30). It is used as a wash to remove the blood from the kidney mince that is prepared for the trypsinization process (31) for the culturing of monolayer cells.

Trypsin Solution. A solution of 0.25 per cent Difco Trypsin 1-250 in

trypsin buffer is used to trypsinize kidney cells in the preparation of cells for monolayer tissue culture.

Sodium Bicarbonate Buffer Solution. A stock solution of 2.8 per cent of sodium bicarbonate is made to buffer all media used for monolayer and suspended type cultures.

Tissue Preparation. Normal monkey kidney cells were used throughout the study. The kidneys were obtained from tuberculin-negative *Macaca rhesus* or *Macaca cynomologus* monkeys. Both monolayer and mince type tissue cultures were used. Youngner's (31) method of trypsinizing cells was used for preparing cells for the monolayer cultures.

Method for Screening the Milk Hydrolysates. A colorimetric assay test for titrating virus as outlined by Salk, Youngner and Ward (32) was adapted for use in comparing the toxicity of the milk hydrolysates to monkey kidney cells. The hydrolysate concentrations tested were from 0.1 to 50.0 g/l. The test is based on a change of the pH of the medium which is dependent on the viability of the cell and necessitates a delicately balanced buffering system, plus a color indicator. If the cells remain viable, their metabolic products will cause the medium to become acid which changes the color of the medium. Destruction or inhibition of the cells causes no color change. The test may be read visually on or after the seventh day. The indicator, phenol red, turns yellow in acid, orange in neutral and red in the alkaline state.

Method for Determining the Optimum Concentration of Test Hydrolysate. The method used to determine the optimum concentration of lactalbumin hydrolysate for cell growth was the Roller Tube Growth Test. This is a test in which trypsin dispersed cells are suspended in the test medium at 1-600 dilution, and 2.0 ml of this suspension are placed in each roller tube. The roller tube cultures are incubated at 37°C. for seven days in a stationary position after which time the cells are observed for proliferation and toxicity. After a change to fresh medium containing additional sodium bicarbonate, cultures are placed on a roller drum and incubated again for seven more days at the same temperature. On the fourteenth day the cells are again observed for viability and a majority of the tubes receive a change to fresh medium inoculated with virus. The remaining tubes also receive a medium change, the fresh medium contains no virus. All the tubes are incubated again for seven days at 37°C. At the end of this period, virus is harvested from several tubes and titrated. The uninoculated tubes are checked for toxicity and serve as controls.

The following media were tested: Medium 199, Medium 199 without the amino acid fraction, various Maximum Test Media which contained 0.1,

0.2, 0.3, 1.0, 3.0, 7.0, 10.0, 20.0 and 30.0 g/l of Edamin, and Minimum Test Media which contained 1.0, 5.0 and 10.0 g/l of Edamin.

Method Used for the Final Comparison of Virus Yields in the Test Media and Control Medium. Virus harvests from bottle cultures of monolayer tissue type cultures and suspended tissue type cultures grown in the test and control media were compared. Stoppered with specially compounded white stoppers known to be non toxic, sixteen ounce prescription type bottles were used for these cultures.

The following procedures were used for each type of culture:

A. *Monolayer cultures.* Kidney cells were prepared by the trypsin process used by Youngner (31). Test and control media buffered with 1.12 g/l of sodium bicarbonate were inoculated with cells to make a 1-600 suspension. Forty ml of each of these cell suspensions were planted in each of 10 prescription type bottles. The bottles were placed flat in a stationary position position for six to seven days at 37°C. The cell sheaths were observed at the end of this period, the old medium was drawn off and 80 ml of fresh medium, buffered with 1.68 g/l of sodium bicarbonate and inoculated with 0.02 ml of seed virus, was added to the bottles. Bottle cultures were incubated at 37°C. for three days and the virus harvested from each individual bottle and titrated.

B. *Suspended Type Cultures.* Kidney cells were prepared by mincing to approximately 1 mm size with sterile barber shears. The cells were then distributed equally into prescription type bottles with 40 ml of either test or control media containing 2.0 g/l of sodium bicarbonate. The bottle cultures were then placed on an arc type rocker and incubated at 37°C. for six days. The medium was changed and 80 ml of either test or control media containing 2.0 g/l of sodium bicarbonate were added to each of the bottles along with 0.02 ml of seed virus. The cultures were then returned to the rockers and rocked at 37°C. for four more days. The virus from each individual bottle was harvested and titrated.

Criteria for Toxicity. Normal monkey kidney cells are fusiform in shape and the cytoplasm appears hyaline. If the medium is not toxic, the tissue cultures of monolayer cells will produce luxuriant sheaths of fusiform cells with hyaline cytoplasm. When cells are exposed to toxic materials in the medium, rounded, abnormally shaped cells, with pyknotic nuclei and granular cytoplasm form. These usually die and disintegrate. When cells are deficient in nutrients the center of the cells become distended laterally, the ends of the cells shrink unevenly tending to form long thin processes and the cytoplasm remains hyaline.

Criteria for Proliferation of Cells in Monolayer Type Tissue Cultures. The trypsin dispersed cells adhere to the glass surfaces of the culture vessels

and form a luxuriant sheath of growing cells within six or seven days. The medium will become acid from the metabolic products. If the cell proliferation is inhibited by toxic materials or deficient nutrients, the cells either do not adhere to the glass surfaces or slowly form incomplete sheaths of abnormal looking cells.

Criteria for Virus Multiplication. A. *Visual.* Virus infection and multiplication is observed through the cytopathogenic phenomena reported by Enders, Weller, and Robbins (1).

B. *Numerical.* At the time of virus inoculation of the tissue cultures, samples of all virus inocula were taken and stored at 5°C. in the tissue culture medium to be tested. The viruses harvested from these cultures were then compared with the above samples to show that the viruses had multiplied and that the test medium was not inhibitory to them.

Method of Assaying Virus. Medium 199, 2 per cent horse serum, and sufficient trypsinized cells to make a 1-600 suspension of cells were dispersed in 2 ml quantities into 15x150 mm Kimble screw cap tubes. These were tightly stoppered and incubated at 36°C. in a stationary position. Cells were observed at the end of seven days and selected for virus titrations after a good monolayer of cells appeared on the glass surface of the tube. Serial ten-fold dilutions of the virus were made up. Quantities of 0.5 ml of each dilution were added to each of ten roller tubes and 1.5 ml Medium 199, containing 1.68 g/l of sodium bicarbonate, were added to all the tubes. Stoppered with specially compounded white rubber stoppers known to be low in toxicity, the tubes were placed on roller drums and incubated at 37°C. for six days. The tubes were read on the third and sixth days for cytopathogenic damage. Fifty per cent Tissue Culture Infectious Dosages ($TCID_{50}$) were calculated for each series of Media by the Reed and Munch method (33). $TCID_{50}$ is the term used by virologists to indicate the concentration of the virus inoculum required to cause cytopathogenic damage to 50% of the roller tube cultures of monolayer cells.

Method of Evaluating the Results. Fiducial limits of the mean with the probability of 95% confidence were computed for the purpose of comparing the experimental and control groups. The formulae are those from Snedecor (34).

RESULTS

The Toxicity of Various Concentrations of Hydrolysates of Milk Proteins on Monkey Kidney Cells

The following hydrolyzed products that were tested to detect toxicity are listed in the order of least toxicity to monkey kidney cells. Edamin, Hycase,

Bacto-Casamino acid, Bacto-Casitone, N-Z Amine Type A, Hycase "Salt Free," Peptonized Milk, N-Z Case, N-Z Amine Type B, and N-Z Amine Type B Batch 668. The cells were able to tolerate up to 50 g/l of Edamin, 20 g/l of Hycase and Bacto Casamino Acid, 10-30 g/l of Bacto-Casitone, and 10 g/l for the remaining products, without showing toxicity. These results are illustrated in Fig. 1 and Fig. 2 where the pH at the termination of the test is below 7.0, the hydrolysates being non-toxic to the cells at those concentrations. The cells showed toxicity in the vitamin-free product (Hycase "Salt Free") at 10.0 g/l, as compared with the cells in the similar hydrolysates containing vitamin components which were toxic at 30.0 g/l. The cells tolerated four times as much Hycase and Bacto-Casamino Acid as the

Figure 1

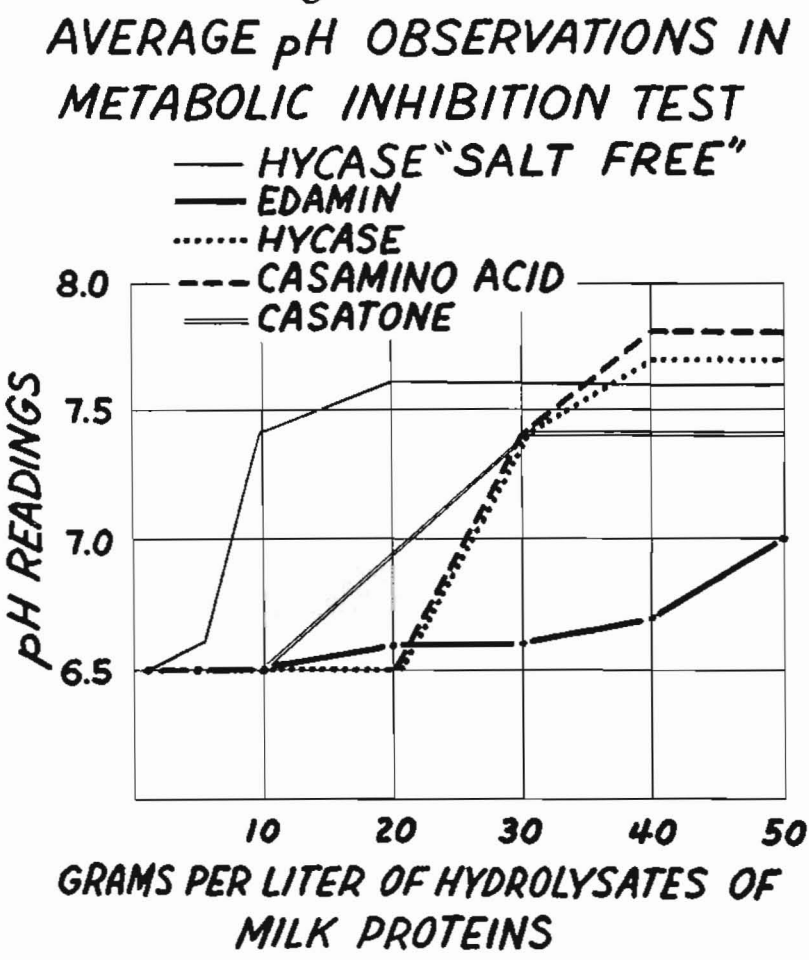
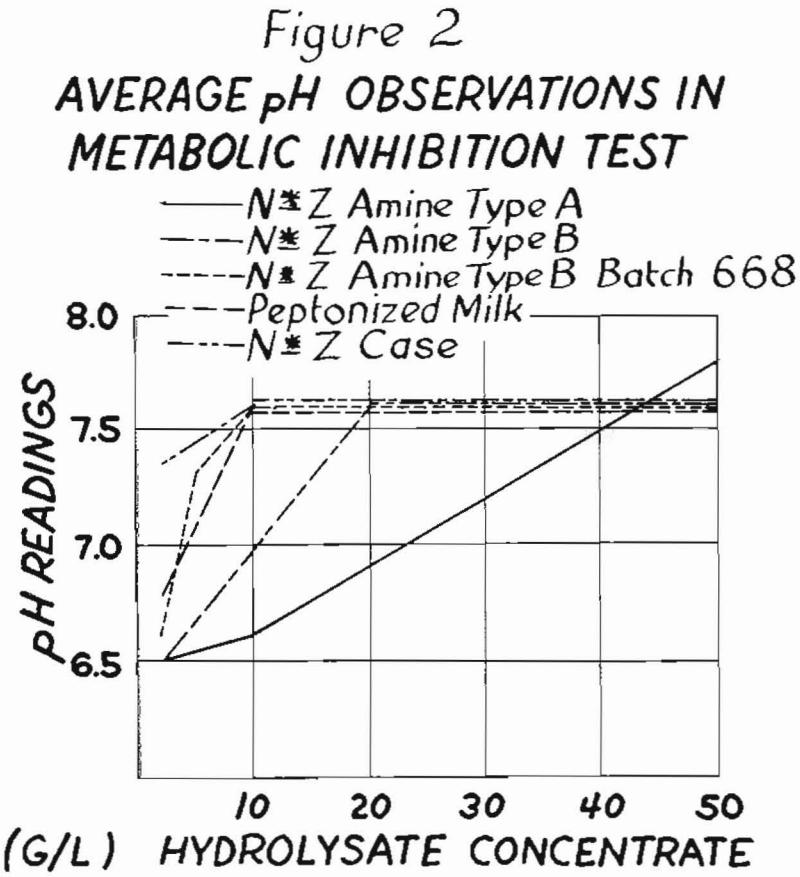


Figure 2



Hycase "Salt Free" product in 5 per cent solutions, as is shown in Fig. 1. Peptonized Milk, N-Z Case, N-Z Amine Type B, and N-Z Amine Type B Batch 668, showed toxicity to the cells in concentrations of less than 10.0 g/l, as is shown in Fig. 2. These products were found to be much more toxic to the cells than were those products such as N-Z Amine Type A and hydrolysates illustrated in Fig. 1.

Since Edamin was found to be the least toxic of all the products tested, it was chosen for further study.

The Effect of Replacing the Amino Acid Fraction in Medium 199 with Various Concentrations of Edamin on Monkey Kidney Cells and Virus Yields Obtained from These Cells

The effect of replacing the amino acid fraction in Medium 199 with Edamin results in an improved medium, since cell growth is more rapid,

cell sheaths are more luxuriant and greater virus yields are obtained than in the original medium. Cells grown in media with 1.0, 3.0, 7.0 or 10.0 g/l of Edamin the latter replacing the amino acid fraction of Medium 199, produced a good sheath of cells in less than seven days and remained in good condition for at least twenty-one days. Virus yields obtained from cultures using 1.0, 3.0, or 7.0 g/l are significantly better than Medium 199 by one log difference in virus titer.

Monkey kidney cells did not produce monolayer cultures in Medium 199 in which the amino acid fraction was left out, or in a medium containing only this fraction, or in the maximum test media containing 20 or 30 g/l of Edamin.

The results of the virus yields obtained from cultures using these media are shown in Table 1.

The Effect of Media Containing Edamin and Balanced Salt Solution on Cultures of Monkey Kidney Cells and the Virus Yields Obtained from These Cultures

The results from a limited number of tests in roller tube cultures indicated that Edamin in concentration between 1.0 and 10.0 g/l contained sufficient nutrients to stimulate monkey kidney cells to proliferate into monolayer cultures that supported good multiplication of Poliomyelitis virus. These roller tube cultures produced greater virus yields than roller tube cultures of Medium 199. (Table 2.)

Monolayer and suspended type bottle cultures using this minimal medium containing 5.0 g/l of Edamin, produced similar growths as those in Medium 199. Virus yields obtained from these cultures were no different than those obtained with Medium 199. Virus obtained from monolayer cultures have titers higher than those obtained from suspended type cultures. This difference in titer is significant with cultures using the hydrolysate medium, but it is not significant with cultures using Medium 199. (Table 3.)

Cultures using Medium 199 appeared to show some signs of degeneration after fourteen days. Cultures using the minimal medium appeared similar in appearance to Medium 199 cultures, while cultures using the maximum medium, in concentrations of Edamin from 1.0 to 7.0 g/l, showed no degenerative changes and appeared good through the twenty-first day.

DISCUSSION

The results obtained using Medium 199 as a culture medium indicate that the nutritional requirements of cells for cultivating viruses may be less exacting than those needed for maintaining cellular activity for extended periods. Cell cultures using this medium begin to show slight signs of de-

generation at fourteen days. This indicates that this medium is not complete in all the essential nutrients required by the cell for maintenance as has been shown for this medium by previous investigators. Cells proliferate to a full sheath of cells, but appear to undergo degeneration when left in Medium 199 for extended periods. Since adsorption studies on Poliomyelitis virus and monkey kidney cells have shown that the virus is adsorbed in fifteen minutes, and can produce and release virus in three to four hours, the condition of the cell at the time of virus infection would seem of more importance than the capacity of the medium to continue the support of the cell. Therefore, a knowledge of substances known to stimulate cell growth and proliferation is more useful in the development of a successful medium for virus cultivation in tissue culture than substances that will prolong the life of the cell for extended periods.

Accelerated rates of growth in cells are made possible by the introduction of additional organic materials such as amino acids, nucleo-proteins, peptids, and coenzymes. Since the anaphylactic properties of proteins and especially those from horse serum are well known, other substances known to contain growth promoting substances which lack anaphylactic properties, should be considered for media used in cultivating virusues for vaccines. Non-protein substances with these characteristics are the hydrolysates of proteins.

Contrary to the findings of the early investigators, the results here presented seem to show that the lower molecular weight products of proteins are more suitable for tissue culture of cells than are the larger molecular weight degradation products. Although this seems to be in conflict with earlier investigations, the difference in the findings may possibly be attributed to the action of enzymes known to be present in the nutrients such as serum and plasma used in the early type cultures. It would seem reasonable to assume that these enzymes brought about the breakdown of the high molecular weight products to lower degradation products which then were readily available to the cells. Thus the early work can be reinterpreted to agree with the results obtained in this investigation.

The hydrolysate of lactalbumin appeared to be tolerated by monkey kidney cells better than the hydrolysates of casein tested. Two of the more important amino acids, cystine and tryptophane, have been found to be lacking in the hydrolysates of casein. Although Rose (35) considered tryptophane but not cystine as one of the essential amino acids for animal nutrition, both of these amino acids have been found essential in tissue culture studies and bacteriological studies by many investigators. It has been shown that the vitamins, pyridine and pantothenic acid, are in lower concentrations in casein hydrolysate than in Edamin, the lactalbumin hydrolysate. Both of these vitamins have been shown to be important as growth stimulants in various plant and animal studies. These differences in the components between Edamin and the casein hydrolysates might be one explanation why Edamin

proved better than the casein hydrolysates in the tissue culture of monkey kidney cells.

The reasons for the success in obtaining greater virus yields by replacing the amino acid fraction in Medium 199 with Edamin have not been determined, but it is believed that increased virus yields are due largely to the production of a greater cell population sensitive to virus infection. Edamin, when incorporated as sole nutrient in a balanced salt solution appears to have sufficient nutrients to supply the amino acids, vitamins and other growth factors necessary for cell proliferation and is equal to the complex Medium 199 for the tissue cultivation of viruses. The minimal medium might be improved by including ribonucleic acid, desoxyribonucleic acid and by increasing the pyrimidines and purines, substances all of which have been shown in phage studies to have improved the host-virus system (36, 37, 38, 39). Ribonucleic acid is believed to be important in multiplication of the virus. The growth of the virus within a cell is accompanied by a rapid increase in ribonucleic acid content (36). Desoxyribonucleic acid is also a constituent of the new virus particles, about two-thirds of it being obtained from the components of the medium (37). According to Weed and Cohen (38) host-cell pyrimidines are utilized directly for viral synthesis and Putman (39) has shown that host purines are used intact for synthesis of virus nucleic acid. Since the Maximum medium contained ribonucleic acid, desoxyribonucleic acid, pyrimidines and purines, it would seem that these components are also important for Poliomyelitis virus in the host-virus system and it is likely that better results were obtained with the Maximum medium because of the presence of these substances.

The success in the cultivation of Poliomyelitis virus in tissue culture with the Edamin media, suggests many uses for these media. The Minimum medium might be considered for the culturing of virus for Poliomyelitis vaccine. Although further testing and approval of the government to substitute this medium for Medium 199 would be required before considering the use of Edamin Medium for vaccine purposes, the reduced cost of this medium, the simplicity of its composition, the accessibility of its components, and the virus yields obtained would probably justify the expenditures required to complete these studies. The pharmaceutical industries that manufacture Poliomyelitis vaccine could also use the Maximum Medium for testing the safety of the vaccine in tissue culture. The results suggest that use of the Edamin Maximum Medium for a culture medium would improve these tests since tissue cultures using this medium are more sensitive to virus infection and the condition of the tissue cells is so much better than that of cells grown in Medium 199. These media might also be used in pH Test, which is used for the potency testing of the vaccine, since it would eliminate the use of horse serum which is sometimes toxic to the cells and causes nonspecific reactions which hamper the reading of the potency test results.

TABLE 1

VIRUS YIELDS FROM ROLLER TUBE CULTURES USING MAXIMUM TEST MEDIA

HYDROLYZATE CONTENT IN MEDIA	NUMBER OF REPLICAS	FIDUCIAL LIMITS ($X \pm S \times t\ 0.05$)
0.1	4	5.5 ± 0.91
0.2	4	6.2 ± 0.59
0.3	4	6.6 ± 0.67
1.0	9	7.3 ± 0.19
3.0	2	7.6 ± 1.27
7.0	5	7.3 ± 0.35
10.0	6	6.7 ± 0.59
20.0	3	NO CELLS
30.0	3	NO CELLS
CONTROL—199 MEDIUM	10	6.5 ± 0.27

TABLE 2

VIRUS YIELDS FROM ROLLER TUBE CULTURES USING MINIMUM TEST MEDIA

HYDROLYZATE CONTENT IN MEDIA (GRAMS/LITER)	NUMBER OF REPLICAS	FIDUCIAL LIMITS $X \pm S \times t\ 0.05$
1.0	2	6.6 ± 0.64
5.0	2	7.7 ± 1.27
10.0	2	7.5 ± 1.93
CONTROL MEDIA		
199 Medium	10	6.5 ± 0.27
199 Medium Without Amiuo Acid Fraction	3	No Cells

TABLE 3

VIRUS YIELDS FROM BOTTLE CULTURES

NUTRIMENT	TYPE OF CULTURE	NUMBER OF REPLICAS	FIDUCIAL LIMITS ($X \pm S \times t\ 0.05$)
1. Minimal Test Medium	M	10	7.7 ± 0.23
2. Medium 199	M	5	7.4 ± 0.36
3. Amiuo Acid Fraction in Medium 199	M	12	No Cells
4. Minimum Test Medium	S	14	7.1 ± 0.28
5. Medium 199	S	10	7.1 ± 0.24

M—Monolayer Cultures

S—Suspended (Mince) Type Cultures

SUMMARY

1. A study was made of ten different hydrolyzed products of milk proteins for their toxicity to monkey kidney cells. Edamin, a hydrolysate of lactalbumin, proved to be the least toxic. The cells could tolerate two and a half times as much Edamin as Hycase and Casamino acids, both hydrolysates of casein which were the next two showing least toxicity.

2. It was shown that Edamin could provide sufficient nutrients to replace the amino acid fraction in Medium 199. Cells in media containing 1.0, 3.0, 7.0 and 10.0 grams of Edamin per liter, produced a good sheath of cells in monolayer tissue type cultures in seven days and remained in good condition, with little or no toxicity, for twenty-one days.

3. Poliomyelitis virus yields obtained from monkey kidney cells grown in media similar to Medium 199, but in which the amino acid fraction of Medium 199 has been replaced with 1.0, 3.0 or 7.0 g/l Edamin, were significantly better than Medium 199 in monolayer tissue type cultures.

4. Roller tubes of monolayer cultures grown in media containing 5.0 or 10.0 g/l Edamin in Hank's Salt Solution, produced Poliomyelitis virus yields that were significantly better than Medium 199.

5. Bottle cultures, usiug a minimal test medium containing only 5.0 g/l Edamin in Hank's Salt Solution, produced suspended type and monolayer tissue cultures that produced Poliomyelitis virus yields equal to those obtained with Medium 199.

6. The practical aspects and the implications of these findings to cell nutrition are discussed.

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